STUDIES ON SHELL FORMATION. V. THE INHIBITION OF SHELL FORMATION BY CARBONIC ANHYDRASE INHIBITORS [1, 2]

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The formation of mollusc shell involves the deposition of crystalline material, which is largely calcium carbonate, in an organic matrix. The source of the carbonate has been uncertain, although its origin from metabolic CO_2 has been suggested (Robertson, 1941; Sobotka and Kann, 1941). The enzyme carbonic anhydrase is present in the shell-forming mantle tissue of many mollusks (Freeman and Wilbur, 1948); and if the rate-limiting reaction in carbonate deposition were either $CO_2 + H_2O \rightarrow H_2CO_3$ or $CO_2 + OH^- \rightarrow HCO_3^-$, we might expect this enzyme to accelerate deposition (Meldrum and Roughton, 1933). This could be tested by applying carbonic anhydrase inhibitors which should retard carbonate formation. Also, if shell carbonate has its origin in metabolic CO_2, carbonate deposition should be accelerated by supplying the mantle with a substrate that can be readily decarboxylated, provided, of course, that carbonic anhydrase is in excess.

These possibilities have been examined in the oyster, *Crassostrea virginica* (formerly *Ostrea virginica*) by measuring the influence of carbonic anhydrase inhibitors and metabolic substrates on the deposition of radioactive calcium in the shell.

METHODS

Oysters were collected in the area of Beaufort, N. C., and maintained in natural waters near the laboratory for several days prior to use at temperatures between 22.8° and 30.0°. The salinity range was 27.6–35.1 except for the period of one week of very low salinity due to a hurricane and during which the oysters were not used. All experiments were carried out within the range 21.5°–25.6°. Individual oysters were prepared as previously described (Wilbur and Jodrey, 1952) and placed in large flat dishes of aerated sea water containing carbonic anhydrase inhibitors for periods of 40 minutes or 7–8 hours. Ca^{45} (4 μc./l.) of high specific activity was then added to the sea water, and the activity of Ca^{45} deposited in the posterior and central regions of the inner shell surface was measured directly (Wilbur and Jodrey, 1952). Isolated mantle preparations with the attached shell (Hirata, 1953; Jodrey, 1953) were employed in studying the effect of respiratory substrates on Ca^{45} deposition both in the presence and absence of carbonic anhydrase inhibitors. Experimental details are presented in the legends of tables.

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Carbonic anhydrase activity was measured in mantle homogenates by the method of Roughton and Booth (1946) as modified by Wilbur and Anderson (1948). Carbonic anhydrase inhibitors were supplied through the kindness of Dr. Emmanuel Waletzky, American Cyanamid Co. The pH of all solutions was adjusted to that of sea water (*ca.* pH 8).

Respiratory measurements on mantle tissue were carried out in oxygen in 5-ml. Warburg flasks using strips of whole mantle weighing 150–220 mg. The thickness of this tissue is such that gas diffusion is not limiting.

Results

Effect of carbonic anhydrase inhibitors on calcium deposition

The effects of 2-benzothiazolesulfonamide (Cpd. I) and 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (Diamox) (Cpd. II) on the deposition of Ca^{45} are given in Table I. Treatment of oysters with Cpd. I at a concentration of 1 : 20,000 and 1 : 50,000 for 40 minutes prior to the addition of Ca^{45} gave essentially complete inhibition of calcium deposition as shown by the ratio of the deposition with the inhib-

Table I

Effect of carbonic anhydrase inhibitors on Ca^{45} deposition in the oyster

	Ratio $\frac{\text{Ca depos.-treated}}{\text{Ca depos.-untreated}}$	Counts/min.		P
		Untreated	Treated	
2-benzothiazole-sulfonamide (Cpd. I)				
1:20,000*	0.02	912±193(12)	41±23(12)	<0.001
1:50,000*	0.07			
1:40,000	0.02	1129±345(8)	43±14(8)	<0.01
1:80,000	0.09			
1:150,000	0.26	1137±261(12)	407±119(11)	<0.05
1:160,000	0.68			
1:250,000	1.11	1189±197(4)	1315±258(4)	<0.8
2-acetylamino-1,3,4-thiadiazole-5-sulfonamide				
1:12,000*	0.60	1243±242(8)	727±178(8)	<0.2
1:25,000*	0.56			
1:100,000	0.52	1550±269(7)	923±219(8)	<0.1
1:150,000	0.71			

Each oyster, connected to a kymograph, was placed in one liter of aerated sea water containing inhibitor. After 40 minutes (starred) or 7–8 hours treatment with the inhibitor 4 μc. Ca^{45} were added. The oyster remained in the radioactive solution for 5 hours. The radioactivity of circular areas of 6.2 cm.^{2} in diameter on the inner surface of the right valve was measured directly. The radioactivities of a posterior and a central area were averaged to give the value for each individual. The means and standard deviations are shown in the columns under the heading counts/min. The numbers in parenthesis in these same columns indicate the number of individuals. Not all the concentrations of Cpd. II for which data were obtained are given in the table. See text concerning statistical procedures.

itor and in its absence (column 2). With a pretreatment of 7–8 hours, a concentration of 1 : 80,000 caused nearly complete inhibition. At 1 : 160,000 inhibition was still marked but it was not present at 1:250,000. Cpd. II also inhibited calcium deposition. Quantitatively, however, the results are quite different with this compound in that the ratio of deposition with the inhibitor and in its absence fell within the range 0.52–0.71 for all concentrations between 1:12,000 and 1:150,000. As the concentration was increased above 1 : 150,000 the maximum effect occurred at 1 : 100,000, and higher concentrations appeared to give no greater inhibition.

Individual differences in the rate of deposition are always very considerable in oysters, and this is shown in Table I, columns 3 and 4. To demonstrate significant differences between treated and control oysters on a statistical basis the concentration groups have been paired. The P values are given in the last column. With Cpd. II the differences between treated and control groups are not highly significant for single concentrations nor for paired groups. However, when all treated and all control groups are combined the difference is significant at the 1% level.

The marked inhibition of carbonic anhydrase by Cpd. I and Cpd. II (Miller, Dessert and Roblin, 1950) has been demonstrated on oyster mantle using supernates of lightly centrifuged homogenates equivalent to one part of tissue (wet weight) in 50 parts of solution. The enzyme activity at 0° was inhibited completely at a concentration of approximately $1:10^7$ for Cpd. I and $1:10^6$ for Cpd. II.

Absence of general toxic action of carbonic anhydrase inhibitors

The inhibition of calcium deposition by Cpd. I and Cpd. II might be expected, quite apart from specific enzyme inhibition, if these compounds had a general toxic action. Accordingly, toxicity of the two carbonic anhydrase inhibitors has been studied at various concentrations by recording shell movements and by measuring the oxygen consumption of the mantle. Kymograph records of shell movements were made for all oysters in each experiment. Movements were normal in Cpd. I at 1 : 80,000 which gave almost complete inhibition of calcium deposition. At 1:50,000 abnormal behavior was shown by more frequent opening and closing of the valves followed by increased periods of closure. This behavior became more marked at higher concentrations. Shell movements remained normal in Cpd. II even at a concentration of 1:12,000. The respiration of mantle was unaffected by either compound at the concentrations shown in Table I. A slight inhibition of oxygen consumption (*ca.* 10%) was produced by both compounds at 1:10,000, however. The results on shell movements and oxygen consumption clearly show that concentrations of the carbonic anhydrase inhibitors which had a marked inhibitory action on calcium deposition exerted no general toxic action on the oyster.

Respiratory substrates on calcium deposition

In the Introduction it was suggested that if shell carbonate is derived from metabolic CO_2, then by supplying substrates that can be decarboxylated, deposition of calcium carbonate should be accelerated. The isolated mantle-shell preparation provides an opportunity to put this to test since substrates can be added directly to the mantle and the deposition of Ca^{45} on the shell measured. Succinate, malate,

and oxaloacetate were used. Previous studies had shown that all three substrates increased the respiration of mantle approximately to the same degree (Jodrey and Wilbur, 1955). Oxaloacetate, in contrast to succinate and malate, is decarboxylated spontaneously and also through the action of the enzyme oxaloacetic decarboxylase found in high concentration in the mantle. Succinate and malate as substrates of the tricarboxylic acid cycle should be converted to oxaloacetate and other compounds which undergo decarboxylation (Jodrey and Wilbur, 1955).

Oxaloacetate was found to increase calcium deposition 4-fold (Table II). The rate of deposition of the isolated mantle was thereby increased approximately to one-half that of the whole oyster. It would be interesting to determine whether a similar effect will result by supplying CO_2 alone. Succinate and malate had no significant effect. The reason for this result is not clear.

TABLE II

Effect of respiratory substrates on Ca^{45} deposition in the mantle-shell preparation of the oyster

Treatment	Ca^{45} depos., counts/min.		P	$\frac{\text{Ca depos.-treated}}{\text{Ca depos.-untreated}}$
	Untreated	Treated		
Succinate	569±91(12)	627±80(12)	<0.7	1.1
Malate	455±67(12)	568±58(12)	<0.3	1.2
Oxaloacetate	533±79(16)	2223±214(23)	<0.001	4.2

Mantle-shell prep. no substrate	521±42(40)	Ratio $\frac{\text{mantle-shell prep.}}{\text{intact oyster}} = 9.4$; $P<0.001$
Intact oyster	5060±88(67)	

Each mantle-shell preparation was placed in 500 ml. of aerated sea water containing the sodium salts of substrates, 0.01 *M*, for 60–75 minutes. Four μc. of Ca^{45} were then added and the preparations remained in the solutions for an additional 11 hours. Radioactivity of the shells and method of expressing data as in Table I. Intact oysters connected to kymographs remained in one liter of sea water containing 4 μc. of Ca^{45} for 5 hours. For comparison with mantle-shell preparations the counts from the shells of intact oysters were multiplied by $2 \times \frac{11}{5}$ to correct for the difference of Ca^{45} activity in the sea water and the time of exposure.

Carbonic anhydrase may be expected to assume increased importance as more CO_2 passes through the system to form carbonate. This should be demonstrated by an increased effect by carbonic anhydrase inhibitors at high calcium deposition rates. To test this the effect of 1:40,000 and 1:80,000 2-benzothiazolesulfonamide was measured with Ca^{45} using the mantle-shell preparation with oxaloacetate added (high deposition rate) and in the absence of added substrate (low deposition rate). In the absence of substrate there was a decreased calcium deposition in the presence of the inhibitor (43% decrease), but the difference between treated and control preparations was not statistically significant ($P < 0.2$; 17 treated specimens; 17 untreated). When oxaloacetate was added, increasing the deposition rate 5.5-fold, the same concentrations of inhibitor produced a significant decrease in calcium deposition amounting to 49% ($P < 0.001$; 16 treated specimens; 23 untreated).

Discussion

The principal findings of this study are a marked increase in calcium deposition when oxaloacetate was made available to the mantle and a marked decrease in calcium deposition produced by carbonic anhydrase inhibitors. A third finding, related to the first, confirms an earlier study (Jodrey, 1953) which reported that the rate of calcium deposition of the isolated mantle-shell preparation is only a fraction of that of the whole oyster. These three results point to certain features of shell formation about which we have had no clear indication heretofore, namely: (1) the origin of shell carbonate; (2) the relation of carbonic anhydrase to shell deposition; and (3) the interrelation of the mantle and the rest of the organism with respect to shell formation. We may now consider these aspects of shell formation briefly.

The increase in calcium deposition which occurred when a source of CO_2 was made available, while somewhat indirect as evidence, strongly suggests that at least a part of the shell carbonate has its origin in the CO_2 of metabolic processes. An alternative source of CO_2 would be the bicarbonate of sea water. This would have to pass into the mantle or other parts of the organism and be converted to CO_2 if carbonic anhydrase were to act as a catalyst. That such a source is important becomes unlikely in view of the low rate of deposition in the isolated mantle-shell preparation immersed in sea water.

The probable importance of carbonic anhydrase catalysis in the conversion of CO_2 to carbonate is indicated by the reduction in the rate of calcium deposition when the oyster is treated with carbonic anhydrase inhibitors. At low rates of calcium deposition, as in the case of the isolated mantle-shell preparation without added substrate, the hydration of the CO_2 is sufficiently rapid without carbonic anhydrase and thus a carbonic anhydrase inhibitor was without significant effect. When the rate was increased by supplying oxaloacetate, however, a clear-cut inhibition occurred. Since the uncatalyzed reaction would always be present, inhibitors should never give complete inhibition. This was the case with experiments using Cpd. II on whole oysters and Cpd. I on the mantle-shell preparation with added oxaloacetate. When whole oysters were treated with Cpd. I, on the other hand, inhibition of calcium deposition was essentially complete. These results point to an inhibition by Cpd. I of factors other than carbonic anhydrase. Such factors are apparently located in parts of the organism other than the mantle since the same inhibition did not occur in the isolated mantle.[4]

Carbonic anhydrase will be important in carbonate formation only when the hydration of CO_2 becomes limiting.[5] Mollusks which are depositing carbonate at a low rate would have no need for the enzyme; and it is not surprising that some mollusks have little or none (Freeman and Wilbur, 1948).

[4] Cpd. I reduced the deposition rate of the whole oyster below that of the mantle-shell preparation. This has no significance for our present discussion, however, since the relative contribution of the mantle *per se* to calcium deposition in the whole organism is unknown.

[5] Theoretically, carbonic anhydrase could also accelerate the solution of shell $CaCO_3$ brought about by the action of weak acids other than carbonic acid, provided that the second of the two following reactions were limiting (Meldrum and Roughton, 1933): $CaCO_3 + 2HA \rightarrow H_2CO_3 + CaA_2$; $H_2CO_3 \rightarrow CO_2 + H_2O$. However, these reactions occur in lamellibranchs when the valves are closed (see Dugal, 1939); and it seems unlikely that the enzyme would be of much importance in this process.

Because the deposition of calcium by the isolated mantle in the absence of substrate is low, it does not follow, of course, that the same low rate obtains in the whole organism. Nonetheless, in view of the marked effect of oxaloacetate on the isolated mantle it seems likely that in the intact organism CO_2 or substrates that can be decarboxylated, or both, are furnished to the mantle and so increase the rate of calcium deposition. Thus, the mantle, while autonomous in one sense, would only perform effectively because the raw materials of shell are furnished by other parts of the organism.

Summary

1. The carbonic anhydrase inhibitors 2-benzothiazolesulfonamide (Cpd. I) and 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (Diamox) (Cpd. II) markedly reduced the rate of deposition of calcium in the shell of the oyster *Crassostrea virginica*. Treatment of oysters with Cpd. I, 1:80,000, for 7–8 hours reduced the rate to one-tenth the normal value and gave essentially complete inhibition at higher concentrations. The latter result indicates that inhibition by this compound is not limited to carbonic anhydrase. With Cpd. II the maximum inhibition was 50% and was produced at a concentration of 1:100,000 or higher.

2. Measurements of shell movements and oxygen consumption of mantle tissue showed that concentrations of carbonic anhydrase inhibitors which had a marked inhibitory action on calcium deposition exerted no general toxic action on the oyster.

3. The rate of calcium deposition of the oyster was more than 9 times that of the isolated mantle-shell preparation, confirming an earlier study. The addition of 0.01 *M* oxaloacetate to the mantle-shell preparation increased the deposition rate more than 4-fold, suggesting the utilization of metabolic CO_2 for shell carbonate by the organism. Succinate and malate were without significant effect on calcium deposition.

4. The experimental findings support the view that carbonic anhydrase increases calcium deposition in the oyster when the rate is sufficiently high that either of the following reactions becomes limiting: $CO_2 + H_2O \rightarrow H_2CO_3$; $CO_2 + OH^- \rightarrow HCO_3^-$. At lower rates the enzyme would not be required.

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